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Human breast cancer prognosis has traditionally been determined by the stage and grade of the primary tumor with lymph node involvement as the major determining factor. For even those with the best prognosis (small, 1 cm diameter-size lesions and no node involvement), there is not a satisfactory method to predict who is in need of additional therapy besides surgery. Of 10 patients who present with this profile, 6-7 will be cured by surgery and 3-4 of them will have progressive disease. We have evaluated two sets of breast cancer cases using protein expression for prognosis. In a pilot study (n=40), we evaluated expression of c-Met and HER2 in primary breast cancers and their lymph node metastases. Neither c-Met nor HER2 expression in primary tumors correlated with established prognostic factors such as age, lymph node involvement, ER, PR, tumor size, or grading. However, c-Met overexpression alone identified high-risk patients independent of HER2. Five-year DFS associated with c-Met overexpressing tumors was 17% compared to 55% in remaining patients (p=0.037; RR 3.0). These results identify c-Met as a target for therapeutic approaches, particularly in HER2 negative patients. We have expanded this to study a series of nearly 200 cases and the analysis of them is still ongoing. The imaging is complete and the correlations will be completed in early 2003.

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Bibliography of Publications and Meeting Abstracts

Manuscript submitted for publication: Relevance of Met, HGF/SF, and HER2 Expression in Primary Breast Cancer and Corresponding Axillary Lymph Node Metastasis Validated by Two Independent Immunohistochemical Assays¹ (Ernst Lengyel, Dieter Prechtel, James Resau, Katja Gauger, Anita Welk, Kristina Lindemann, Georgia Salanti, Thomas Richter, Beatrice Knudsen, George F. Vande Woude, and Nadia Harbeck²)

Meeting abstracts: None

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Introduction

Receptor tyrosine kinases (RTKs) play an important role in malignant transformation of epithelial cells by activating signal transduction pathways important for proliferation, invasion and metastasis. Met is a tyrosine kinase receptor that is expressed in many normal human epithelial tissues and often overexpressed in solid tumors. Met mediates the diverse activities of its ligand, hepatocyte growth factor/scatter factor (HGF/SF), such as proliferation, cell migration, and invasion as well as angiogenesis. HGF/SF also mediates lumen formation and branching morphogenesis in a variety of cell types. In animal model systems, HGF/SF secretion is sufficient for tumorigenesis and metastasis (1,2).

Many tumor types have been found to overexpress Met and HGF/SF. Met overexpression in breast tumors is associated with breast cancer progression (3–5) and high HGF/SF also

correlates with poor survival in ductal breast carcinomas (6,7). Tsarfaty et al. (1999) (4) quantified Met expression in uninvolved (N) relative to tumor (T) tissue in the same primary breast carcinoma section. The overall Met distribution in this patient group was ~40% with $T < N$, ~40% had $N = T$, and 20% had $T > N$. Higher Met expression in tumor than in normal tissue was associated with poor patient outcome. Three groups (8–10) have been examined for both Met and HGF/SF expression in benign and malignant breast tissue. We found frequent expression of the receptor and its ligand and that the expression is higher in breast cancer and carcinomas *in situ* compared to benign breast tissue. While Met was mainly detected in the epithelial breast cancer cells, HGF/SF was detected in tumor cells as well as in stroma cell types implicating that HGF/SF contributes to growth and invasiveness of breast cancer cells by autocrine and paracrine mechanisms. This is also supported by recent experiments showing increased tumorigenic and metastatic activity accompanied by reduced tubule formation of breast cancer cells after transfection with Met and HGF/SF (5).

The HER2 receptor tyrosine kinase is a well-characterized oncogene in breast cancer and is a target for an antibody-based tumor therapy approved for clinical use (11). No study has determined whether the Met is coordinately expressed with HER2 in breast cancer and, if so, whether their co-expression is clinically relevant. Given the important role of Met in tumor invasion and metastasis and the fact that HER2 is only positive in 20–30% of patients with breast cancer (12), we investigated whether Met is co-expressed in HER2 positive tumors. We show that Met overexpression, as independently assessed by two immunohistochemical methods, is associated with significantly diminished disease-free survival (DFS) that is independent of HER2 overexpression.

Body -

Key Research Accomplishments

We collaborated with two groups (in Munich, Germany, and Chicago, U.S.A.) to determine the pattern and characteristics of these important tyrosine kinase receptor proteins in human primary breast cancer. The first was a small group of 40 cases with known clinical outcomes and follow up. This group established the parameters and profiles we would need

to determine the same findings in our second collaborator's series. The second group also has known follow up but we are blinded to that information. The parameters and setting for the pilot study determine the methods for the main study.

In both cases, the receptor tyrosine kinases are well characterized as regards breast cancer. Met and its ligand HGF/SF are potential candidates as targets for breast cancer therapy because of their *in vitro* ability to transform mammary epithelial cells and induce metastasis (2,5). An antibody (Herceptin®) against the HER2 receptor tyrosine kinase has shown clinical utility by improving survival in patients with HER2 positive breast cancer. Yet, only 20–30% of all breast cancer patients show HER2 overexpression and are thus eligible for Herceptin® therapy (12); this leaves the majority of patients without the option to be treated by this approach. Therefore, it is highly desirable to identify and characterize other biological targets in the group of patients, which are not appropriate for therapy with Herceptin®. In a pilot study, we assessed expression and clinical impact of Met and its ligand HGF/SF in primary breast cancer tissue and corresponding lymph node metastasis and compared it to that of HER2. Our results—obtained by two independent immunostaining techniques—substantiate the clinical relevance of Met.

We observed two distinct staining patterns for Met, cytoplasmic and membrane staining. Even though membrane staining seemed to be associated more strongly with tumor aggressiveness, the clinically most informative results were obtained by focusing on total Met expression (cytoplasmic + membrane staining) using either IF or conventional IHC staining. Met overexpression was found both in the primary tumor tissue and in the corresponding lymph node metastasis suggesting an association with tumor progression and metastatic potential. Additional high-risk patients were identified according to Met overexpression in the lymph node metastasis. High expression of the ligand HGF/SF was associated with a better outcome, if it coincided with low Met expression. Although there may be other explanations, perhaps HGF/SF induces cell differentiation and Met downregulation (5). In lymph node metastasis, HGF/SF expression was predominantly lower than in the primary tumor, possibly due to ligand utilization and receptor down modulation. Overexpression of both HGF/SF and Met in the primary tumor was associated

with poor clinical outcome. This is concordant with *in vitro* and *in vivo* data showing that co-expression of the receptor and its ligand leads to increased tumor aggressiveness in a variety of solid tumors. Our data support the hypothesis that Met-HGF/SF signaling, and particularly the expression level of Met, is critical for the balance between cell differentiation and tumorigenicity (5).

The patients evaluated here with more than three involved axillary lymph nodes are high-risk breast cancer patients in whom assessment of the prognostic impact of tumor biological markers may be hampered by effects of adjuvant systemic therapy (18). Nevertheless, even within this group of patients at rather high risk of recurrence, high Met expression in primary tumor was associated with poor prognosis and all relapses in patients with tumors overexpressing Met occurred within the first 14 months after primary therapy, suggesting poor response to adjuvant systemic treatment. Clinical risk-group assessment obtained by Met was different than currently available tumor biological markers. It differed from that found with uPA/PAI-1 (data not shown), which are the only novel tumor markers that have reached the highest level of evidence (LOE I) for clinical utility and which recognize a different group of patients at risk than HER2. Risk group assessment by Met also differed from that of HER2 indicating that Met high-risk patients are not those who are already candidates for Herceptin™ therapy. It is important to note that these clinically relevant findings were independently determined using confocal IF and conventional IHC and scoring algorithms for Met and HER2 expression (14).

In the pilot study, Met overexpression in primary node-positive breast carcinomas appears to correlate with tumor aggressiveness and failure of adjuvant systemic therapy. The differential expression of Met between primary tumor and lymph node metastasis and the correlation with high-risk patients identify Met as a novel target for tumor therapy, whether or not HER2 positive. The results of this analysis are now being evaluated in the larger series of cases. All these cases have been evaluated and imaged for c-Met and Her2neu by laser scanning confocal microscopy. Their digital images have been converted into a variety of data sets (e.g., average intensity, ratio of normal to tumor intensity, DV statistic, etc.). The ranking of cases in a variety of patterns has been completed. These include ranking by

tumor expression, ranking by tumor to tumor ratio, ranking of Met-Her2neu ratios and amongst and between the two by the same categories. These are now being correlated with clinical outcomes by our collaborators and those results will be determined in early 2003 at the latest. A supplemental report with prognostic determinations will be submitted at that time. A draft of our submitted paper for the pilot study is provided (see appendix) and a set of representative images with digital files is also submitted for the larger study.

Reportable Outcomes

The determination of the protein expression of c-Met is a prognostic indicator of human primary breast cancer.

Conclusions

c-Met is an important protein that determines breast cancer prognosis in human carcinomas.

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APPENDIX

(Manuscript Submitted for Publication)

Relevance of Met, HGF/SF, and HER2 Expression in Primary Breast Cancer and Corresponding Axillary Lymph Node Metastasis Validated by Two Independent Immunohistochemical Assays¹

Ernst Lengyel, Dieter Prechtel, James Resau, Katja Gauger, Anita Welk, Kristina Lindemann,
Georgia Salanti, Thomas Richter, Beatrice Knudsen, George F. Vande Woude,
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Keywords: breast cancer, Met, HER2, HGF/SF, prognosis, therapy target

Running title: Met, HGF/SF, and HER2 in breast cancer

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The abbreviations used are: Disease-free survival (DFS); Hepatocyte Growth Factor/Scatter Factor (HGF/SF); Immunohistochemistry (IHC); Immunofluorescence (IF); Receptor tyrosine kinases (RTK).

ABSTRACT

In a pilot study (n=40), we evaluated expression of the Met and HER2 receptor tyrosine kinases in primary breast cancers and their lymph node metastases, comparing conventional immunohistochemistry (IHC) determination with confocal immunofluorescence (IF) imaging. In this study, the small number of HER2 positive cases was not prognostic and neither Met nor HER2 expression correlated with established prognostic factors such as age, lymph node involvement, ER, PR, tumor size, or grading. However, both analytical methods unequivocally showed a correlation of Met overexpression with patients at high risk. Median DFS associated with Met overexpressing tumors was 8 months compared to 53 months in remaining patients ($p=0.031$; RR 3.1). The results by both analytical methods identify Met as a target for therapeutic approaches and may be particularly important in patients negative or positive for HER2.

INTRODUCTION

Receptor tyrosine kinases (RTKs) play an important role in malignant transformation of epithelial cells by activating signal transduction pathways important for proliferation, invasion and metastasis. Met is a tyrosine kinase receptor that is expressed in many normal human epithelial tissues and often overexpressed in solid tumors. Met mediates the diverse activities of its ligand, hepatocyte growth factor/scatter factor (HGF/SF), such as proliferation, cell migration, and invasion as well as angiogenesis. HGF/SF also mediates lumen formation and branching morphogenesis in a variety of cell types. In animal model systems, HGF/SF secretion is sufficient for tumorigenesis and metastasis (1,2; the reference list is found starting on p. 8).

Many tumor types have been found to overexpress Met and HGF/SF. Met overexpression in breast tumors is associated with breast cancer progression (3–5) and high HGF/SF also correlates with poor survival in ductal breast carcinomas (6,7). Tsarfaty et al. (1999) (4) quantified Met expression in uninvolved (N) relative to tumor (T) tissue in the same primary breast carcinoma section. The overall Met distribution in this patient group was ~40% with $T < N$, ~40% had $N = T$, and 20% had $T > N$. Higher Met expression in tumor than in normal tissue was associated with poor patient outcome. Three groups (8–10) have been examined for both Met and HGF/SF expression in benign and malignant breast tissue. We found frequent expression of the receptor and its ligand and that the expression is higher in breast cancer and carcinomas *in situ* compared to benign breast tissue. While Met was mainly detected in the epithelial breast cancer cells, HGF/SF was detected in tumor cells as well as in stroma cell types implicating that HGF/SF contributes to growth and invasiveness of breast cancer cells by autocrine and paracrine mechanisms. This is also supported by recent experiments showing increased tumorigenic and metastatic activity accompanied by reduced tubule formation of breast cancer cells after transfection with Met and HGF/SF (5).

The HER2 receptor tyrosine kinase is a well-characterized oncogene in breast cancer and is a target for an antibody-based tumor therapy approved for clinical use (11). No study has determined whether the Met is coordinately expressed with HER2 in breast cancer and, if so, whether their co-expression is clinically relevant. Given the important role of Met in tumor invasion and metastasis and the fact that HER2 is only positive in 20–30% of patients with breast cancer (12), we investigated whether Met is co-expressed in HER2 positive

tumors. We show that Met overexpression, as independently assessed by two immunohistochemical methods, is associated with significantly diminished disease-free survival (DFS) that is independent of HER2 overexpression.

MATERIAL AND METHODS

Patient specimens: Clinical relevance of Met and HER2 expression was retrospectively evaluated in 40 primary breast cancer patients having three or more positive axillary lymph nodes. Patients received their primary therapy at the Department of Obstetrics and Gynecology of the Technical University of Munich, Germany between 1989 and 1997. Informed consent for analysis of tumor biological factors was obtained before surgery. Treatment decisions were based solely on consensus recommendations at the time. After surgery, 25 patients received adjuvant chemotherapy (the majority CMF), 17 patients endocrine therapy (tamoxifen), and 4 patients received combined chemo-endocrine therapy. Median patient age was 54 years (range: 28–80 years). Patient characteristics are summarized in Table 1; established prognostic factors were dichotomized as described elsewhere (13). At time of primary therapy, no patient had any clinical or radiologic evidence of distant metastases. Follow-up data was obtained in regular intervals (13). Median follow-up in patients still living at time of analysis was 70 months (range: 12–122 months); 21 patients (53 %) experienced disease recurrence, the majority (n=18) at distant sites. In all patients, uPA and PAI-1 antigens have been measured by ELISA (uPA: Imubind # 894. PAI-1: Imubind # 821; both from American Diagnostica, Greenwich, CT) (14) in a *prospective* fashion since 1987 (13–14). Previously determined and re-evaluated optimized cutoff values were used for uPA (3 ng/mg protein) and PAI-1 (14 ng/mg protein) (13,14). The combination of both factors (uPA/PAI-1) was considered low if both factors were low and high if either or both were high (13,14).

Conventional Immunohistochemical staining: Expression of Met, HGF/SF, and HER2 in breast carcinoma tissue and lymph node metastases was determined by immunohistochemistry (IHC) using the following primary antibodies: Met (pAB C28), HGF/SF (pAB; both from Santa Cruz Biotechnology, CA, USA) and HER2 (pAB A0485, Dako, Denmark). A peroxidase-based visualization system was used together with the respective secondary antibodies. As positive controls, an NIH 3T3 cell line stably transfected with Met and HGF/SF (1,16) as well as HER2-overexpressing breast cancer tissue were used. Negative controls were performed by omission of the respective primary antibody. Immunohistochemical reactivity was scored independently by two staff pathologists (D.P. and T.R.) according to the immunoreactive score (IRS) first described by Remmele and Stegner (17). For cytoplasmic Met (Met^{cyt}) and HGF/SF staining, the percentage of stained tumor cells was grouped into four categories: ≤ 10 , ≤ 40 , ≤ 70 , > 70 %. A scale from 0 (no staining) to 3 (intense staining) was assigned to staining intensity. The immunoreactive score from 0 to 12 (17) was assigned by multiplying positivity category times staining intensity. For Met membrane staining (Met^{mem}), a score from 0 (no membrane staining) to 2 (strong membrane staining) was applied. Optimized cutoffs determined by log-rank statistics were used for Met^{cyt} (score 6), Met^{mem} (score 1), and HGF/SF (score 3) to discriminate between high and low immunostaining in the primary tumor tissue. In the lymph node metastases, the respective cutoffs were score 8 for Met^{cyt}, score 1 for Met^{mem}, and score 6 for HGF/SF. Met^{cyt} and Met^{mem} were combined in order to

enable assessment of *total Met* expression; High expression of both Met^{cyt} and Met^{mem} was considered Met overexpression.

HER2 immunostaining was performed and scored (0 to 3+) as done for clinical routine. A score of 3+ was considered HER2 overexpression. Distributions for Met, HGF/SF, and HER2 in primary tumors and axillary lymph node metastases are summarized in Table 2.

Statistical Analysis: Correlations between continuous variables were analyzed using the Spearman rank test. Associations between continuous and/or categorical variables were analyzed using the Mann-Whitney U-test, the χ^2 test, or the McNemar test as appropriate. Continuous variables were coded as binary variables by employing log-rank statistics to determine optimal cutoffs discriminating low and high-risk patients using the statistical programming environment S-Plus (MathSoft 1998) (13). For univariate analysis of disease-free (DFS) survival, Kaplan-Meier curves were plotted and then compared using log-rank statistics. Multivariate analyses were performed in a stepwise forward fashion by applying the Cox proportional hazards model using the SPSS software package (SPSS Inc., Chicago, IL). All tests were performed at a significance level of $\alpha = 0.05$.

Confocal microscopy / Immunofluorescence (IF) analysis: Met and HER2 expression in primary tumors was assessed using an IF analysis staining protocol and a computerized evaluation algorithm as described earlier (4, 15). Breast samples were stained using rabbit polyclonal antibody (pABC28) of Met (Santa Cruz). The indirect staining for Met was done with a rhodamine-labeled secondary antibody against rabbit immunoglobulin. The HER2 (c-neu) antigen was identified using a monoclonal antibody (Oncogene Science, MA). The indirect staining for HER2 was done using a FITC labeled secondary antibody against mouse immunoglobulin. The staining was imaged using a Zeiss 410 confocal microscope and the 8-bit digital images were stored to disk. The individual FITC or Rh images were evaluated using the Media Cybernetics image analysis program, Image Pro (Washington). The images were ranked according to their average intensity using pixels of intensity from 40–255 gray scales from the individual images. The images were ranked by their average intensity and cut-off values were established based upon intensity. We used “secondary only” stained controls as well as positive control cells from a cell line stably transformed with human Met that was prepared into a paraffin block.

The staining was ranked according to tumor intensity, ratio of tumor staining to adjacent, parallel uninvolved normal for those cases with both normal and tumor in the same section as well as against the control samples. The images were evaluated “qualitatively” as well as by image analysis. Those cases above the cut off were scored as positive. The review was independently done by three expert observers as well as by consensus review after individual ranking.

Statistical Analysis: For statistical analysis, the computer-calculated expression data were grouped as follows: for Met and for HER2, stronger expression in tumor than in surrounding normal tissue was considered overexpression (4,15). Lack of normal tissue in some sections did not allow determination of an IF score.

RESULTS

Conventional IHC and confocal IF render concordant results: Both conventional IHC and confocal IF methods were used to analyze breast cancer patient specimens (Figure 1). By IHC, two distinct staining patterns were observed for Met: cytoplasmic and membrane staining (Figure 1). Membrane staining was observed only in tumor cells. Cytoplasmic staining in tumor cells was substantially stronger than in surrounding normal tissue with considerable variations between different tumors, independent of classical morphological criteria such as typing or differentiation. In normal tissue, only weak cytoplasmic staining (score 0–6) was seen, with higher expression observed in non-neoplastic alterations such as apocrine metaplasia, adenosis, and epithelial hyperplasia. In lymphatic tissue, no specific cellular Met staining was found and, with IHC, cytoplasmic staining was seen specific in tumor cells.

Determination of Met overexpression both by conventional IHC and confocal IF rendered concordant results (Figure 1). Strikingly, all tumors with high Met expression by IHC were classified as having stronger Met expression than the adjacent equivalent normal breast epithelial tissue by confocal IF ($T > N$; $r = 0.41$; $p = 0.01$; see Table 3).

Differential expression of Met, HGF, and HER2 in primary tumor and corresponding lymph node metastasis by conventional IHC: A significant correlation between expression levels in primary breast carcinoma tissue and nodal metastasis was only found for Met^{cyt} ($r = 0.39$; $p = 0.017$) and HER2 ($r = 0.59$; $p < 0.001$). HER2 overexpression in the primary tumor coincided with overexpression in the lymph node in 8/9 cases; an additional six cases overexpressed HER2 in the lymph node even though the primary tumor did not.

For Met^{mem} and HGF/SF, expression patterns differed significantly between primary tumor and lymph node: for Met^{mem}, six of the eight cases with overexpression in primary tumor also overexpressed Met^{mem} in the lymph node; in addition, 10/26 cases with low expression in the primary tumor showed overexpression in the lymph node ($p = 0.039$). For HGF/SF, 17/24 cases with overexpression in the primary tumor did not overexpress the ligand in the lymph node; in 12/14 cases with low HGF/SF expression in the primary tumor, low expression was also seen in the lymph node ($p = 0.001$).

IHC Correlations and associations: In primary tumor tissue, HER2 expression levels were significantly though weakly correlated with Met^{cyt} ($r = 0.41$; $p = 0.011$), HGF/SF ($r = 0.36$; $p = 0.028$), and Met^{mem} ($r = 0.36$; $p = 0.024$). In addition, Met^{mem} was significantly correlated with Met^{cyt} ($r = 0.32$; $p = 0.013$) and HGF/SF ($r = 0.35$; $p = 0.033$). However, Met^{mem}, Met^{cyt}, HGF/SF, and HER2 expression levels in the primary tumor were not significantly correlated to established prognostic factors (age, number of involved lymph nodes, tumor size, grading, ER, PR). The only exception was an inverse correlation between HER2 and ER ($r = -0.35$; $p = 0.027$).

Different primary breast carcinomas were characterized by IHC for Met^{cyt} or Met^{mem} overexpression compared to HER2 overexpression: only four of the nine patients with HER2 overexpression also overexpressed Met^{mem}, while an additional seven patients overexpressed Met^{mem} but not HER2. Similarly, six of nine patients with HER2

overexpressing tumors also had Met^{cyt} overexpression, while Met^{cyt} overexpression without HER2 overexpression was found in six patients. In total, only three of the six tumors with Met overexpression also overexpressed HER2. This is concordant with the data obtained by confocal IF analysis: of the seven cases with Met overexpression (T>N), only three overexpressed HER2 (p=0.049).

With regard to Met and its ligand, HGF/SF, all Met overexpressing tumors (n=6) also overexpressed HGF/SF; both markers were low in 14 tumors and in the remaining 18 cases, HGF/SF overexpression was observed with low Met expression (p<0.001).

Survival analysis

Patients with high Met^{mem} by IHC had a median disease-free survival time of 14 months, in contrast to 56 months for the patients with low Met^{mem} expression (univariate Kaplan-Meier analysis). However, while this difference fails significance (p=0.085) when total Met is used. The survival disadvantage associated with Met overexpression was significant (p=0.021): a median disease-free survival time of 8 months was seen in this high-risk group in contrast to 53 months in the remaining patients (Figure 2, right). All relapses in the high-risk group occurred within the first 14 months after primary therapy. Patients with high Met overexpression had a threefold increased risk of relapse (p=0.031; RR 3.1; 95 % CI 1.1-8.6) in univariate Cox analysis. Similarly, patients with Met overexpression as determined by confocal IF (which includes both Met^{cyt} and Met^{mem}) had a significantly worse DFS (5-year DFS 30 %) than those with low Met (5-year DFS 54 %) (p=0.045; RR 2.5; 95 % CI 1.02-6.01) (Figure 2, left). Neither Met^{cyt} alone nor HER2 had a significant impact on DFS. High HGF/SF was associated—though not significantly—with longer DFS. Patients whose tumors overexpressed HGF/SF but not Met had a very good prognosis compared to those with overexpression of both HGF/SF and / or Met (p=0.011) (Figure 3).

Multivariate Cox analysis for DFS was performed including all established factors, adjuvant chemo- and hormone therapy, HER2, HGF/SF as well as Met^{mem} and Met^{cyt} separately and their combination: Met overexpression (p=0.012; RR 4.0; 95 % CI 1.4-11.9) and adjuvant hormone therapy (p=0.026; RR 0.3; 95 % CI 0.1-0.9) were the only significant factors.

DISCUSSION

The receptor tyrosine kinase Met and its ligand HGF/SF are potential candidates as targets for breast cancer therapy because of their *in vitro* ability to transform mammary epithelial cells and induce metastasis (2,5). An antibody (Herceptin[®]) against the HER2 receptor tyrosine kinase has shown clinical utility by improving survival in patients with HER2 positive breast cancer. Yet, only 20-30% of all breast cancer patients show HER2 overexpression and are thus eligible for Herceptin[®] therapy (12); this leaves the majority of patients without the option to be treated by this approach. Therefore, it is highly desirable to identify and characterize other biological targets in the group of patients which are not appropriate for therapy with Herceptin[®]. In a pilot study, we assessed expression and clinical impact of Met and its ligand HGF/SF in primary breast cancer tissue and corresponding lymph node metastasis and compared it to that of HER2. Our results—obtained by two independent immunostaining techniques—substantiate the clinical relevance of Met (Table 3).

We observed two distinct staining patterns for Met, cytoplasmic and membrane staining. Even though membrane staining seemed to be associated more strongly with tumor aggressiveness, the clinically most informative results were obtained by focusing on total Met expression (cytoplasmic + membrane staining) using either IF or conventional IHC staining (Figure 2) as previously shown in confocal IF (4). Met overexpression was found both in the primary tumor tissue and in the corresponding lymph node metastasis suggesting an association with tumor progression and metastatic potential. Additional high-risk patients were identified according to Met overexpression in the lymph node metastasis. High expression of the ligand HGF/SF was associated with a better outcome, if it coincided with low Met expression. Although there may be other explanations, perhaps HGF/SF induces cell differentiation and Met downregulation (5). In lymph node metastasis, HGF/SF expression was predominantly lower than in the primary tumor, possibly due to ligand utilization and receptor down modulation. Overexpression of both HGF/SF and Met in the primary tumor was associated with poor clinical outcome. This is concordant with *in vitro* and *in vivo* data showing that co-expression of the receptor and its ligand leads to increased tumor aggressiveness in a variety of solid tumors. Our data support the hypothesis that Met-HGF/SF signaling, and particularly the expression level of Met, is critical for the balance between cell differentiation and tumorigenicity (5).

The patients evaluated here with more than three involved axillary lymph nodes are high-risk breast cancer patients in whom assessment of the prognostic impact of tumor biological markers may be hampered by effects of adjuvant systemic therapy (18). Nevertheless, even within this group of patients at rather high risk of recurrence, high Met expression in primary tumor was associated with poor prognosis and all relapses in patients with tumors overexpressing Met occurred within the first 14 months after primary therapy, suggesting poor response to adjuvant systemic treatment. Clinical risk-group assessment obtained by Met was different than currently available tumor biological markers: it differed from that found with uPA/PAI-1 (data not shown), which are the only novel tumor markers that have reached the highest level of evidence (LOE I) for clinical utility (14) and which recognize a different group of patients at risk than HER2 (19, 20). Risk group assessment by Met also differed from that of HER2 indicating that Met high-risk patients are not those who are already candidates for Herceptin™ therapy. It is important to note that these clinically relevant findings were independently determined using confocal IF and conventional IHC and scoring algorithms for Met and HER2 expression.

In conclusion, Met overexpression in primary node-positive breast carcinomas appears to correlate with tumor aggressiveness and failure of adjuvant systemic therapy. The differential expression of Met between primary tumor and lymph node metastasis and the correlation with high-risk patients identify Met as a novel target for tumor therapy, whether or not HER2 positive.